Developmental Cell
Article

Clathrin-Mediated Internalization Is Essential for Sustained EGFR Signaling but Dispensable for Degradation

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DOI 10.1016/j.devcel.2008.06.012

SUMMARY

Clathrin-mediated endocytosis (CME) is the major pathway of epidermal growth factor receptor (EGFR) internalization. It is commonly believed that CME mediates long-term attenuation of EGFR signaling by targeting the receptor for degradation. However, the EGFR can also be internalized through (a) clathrin-independent pathway(s), and it remains unclear why distinct mechanisms of internalization have evolved. Here, we report that EGFRs internalized via CME are not targeted for degradation, but instead are recycled to the cell surface. By contrast, clathrin-independent internalization preferentially commits the receptor to degradation. This finding has profound implications for signaling, as by skewing EGFR fate toward recycling rather than degradation, CME prolongs the duration of signaling. Our data show that CME determines the longevity of some EGFR-activated signaling pathways and that EGF-dependent biological responses, such as DNA synthesis, absolutely require CME. Thus, CME of the EGFR unexpectedly has a greater impact on receptor signaling than on receptor degradation.

INTRODUCTION

Receptor tyrosine kinases (RTKs) signal through a variety of mechanisms, thereby controlling numerous cellular functions. Endocytosis is a major mechanism of RTK signal attenuation, as it permits the removal of RTKs from the cell surface. A wealth of evidence (von Zastrow and Sorkin, 2007), stemming from the initial observation that inhibition of internalization also decreases certain signaling mechanisms (Vieira et al., 1996), has however dramatically changed our perception of endocytosis. Indeed endocytosis is an integral part of signaling, not just a mechanism of attenuation, and it provides spatial and temporal dimensions to signaling events. Thus, a detailed molecular description of how the endocytic and the signaling programs are written into each other is indispensable for our understanding of cell regulation.

The epidermal growth factor (EGF) receptor (EGFR) is one of the best-characterized RTKs. A major mechanism of internalization of EGFR is clathrin-mediated endocytosis (CME), whereby the receptor is removed from the surface via clathrin-coated pits and then routed to the early endosomes. Here, the receptor fate is decided: either it is recycled to the cell surface, or it is further routed for degradation to late endosomes and lysosomes. In recent years, a spectacular series of studies has clarified many molecular aspects of this complex circuitry [reviewed in (Kaksonen et al., 2006; Maldonado-Baez and Wendland, 2006; Mukhopadhyay and Riezman, 2007; Soldati and Schliwa, 2006)].

Another route of internalization of the EGFR (Orth et al., 2006; Sigismund et al., 2005), and of other plasma membrane proteins (Mayor and Pagano, 2007), does not depend on the clathrin pathway. The molecular nature of this "alternative" pathway is ill-defined (Mayor and Pagano, 2007; Parton and Simons, 2007). Essentially the pathway is characterized by its insensitivity to functional ablation of clathrin, a fact that led to its rather non-descriptive definition of non-clathrin endocytosis (NCE). NCE is also sensitive to cholesterol-interfering drugs; since cholesterol is enriched in membrane rafts, the pathway is sometimes referred to as "raft-dependent". Finally, since caveolae (another endocytic organelle, characterized by the presence of caveolin-1 and by the absence of clathrin) are a form of raft-like structure (Parton and Simons, 2007), the pathway is sometimes referred to as "caveolar" or "raft/caveolar". This rather blurry picture reflects our poor understanding of the molecular mechanisms that regulate NCE, an issue compounded by the fact that it is not at all clear whether NCE is a single pathway or, as appears more likely, a mixture of pathways, possibly with partially overlapping characteristics (Mayor and Pagano, 2007).

Despite these difficulties an operational definition can be attempted. Herein, we refer to NCE with the following explicit assumptions: (1) our operational definition applies only to the receptor system under study, i.e., the EGFR; this does not imply that other receptors are not internalized through NCE, but simply that generalizations should be avoided, considering the present state of knowledge; (2) NCE is defined, for experimental purposes, by its resistance to clathrin ablation; (3) NCE is sensitive to the cholesterol-interfering drug filipin (see below); and (4) regardless of whether NCE is the product of several pathways, they all obey the above requirements. By using this definition, we have previously shown that the EGFR is internalized both through CME and NCE as a function of ligand dose (Sigismund et al., 2005). In particular, at low doses of EGF, the EGFR is preponderantly internalized through CME; at higher doses, the

receptor is internalized through both CME and NCE (Sigismund et al., 2005). In the same study, we provided evidence that receptor ubiquitination is indispensable for NCE of the EGFR. Thus, this requirement might be added to the definition of EGFR uptake via NCE.

Here, we endeavored to assess the physiological relevance and the cellular function(s) of the two pathways of EGFR internalization. We explored their impact on EGFR internalization, fate, and signaling. We selectively analyzed CME-mediated events by stimulating the receptor with low doses of ligand, a condition in which CME is the predominant pathway of internalization. Conversely, we honed in on NCE-associated events under conditions of high EGF in cells in which CME was specifically inhibited through functional ablation of clathrin or of the major clathrin adaptor AP-2. Additional pharmacological (filipin) or molecular (EGFR mutants) tools were also used to further corroborate our conclusions. By exploiting this multifaceted approach, we report here that CME does not play a major role in EGFR degradation, but rather in its recycling to the cell surface in order to maintain sustained signaling. On the other hand, EGFRs internalized through NCE are efficiently degraded. Thus, the two routes of EGFR internalization have profoundly different effects on EGFR fate and signaling abilities.

RESULTS

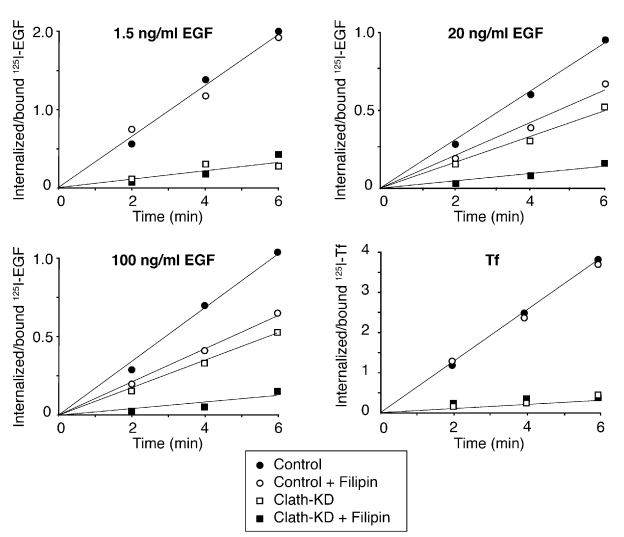
CME Is Not the Major Pathway of EGFR Degradation

To analyze the relevance of CME to EGFR fate, we used HeLa cells in which clathrin was functionally ablated by siRNA-mediated knockdown (KD). Three different clathrin silencing oligos, with appropriate mismatched controls, were used with comparable results. Data obtained with one oligo (# 1) are shown in the main text, whereas representative data obtained with the other oligos are shown in Figure S1 (available online). In addition, re-expression of clathrin in clathrin-KD cells could reconstitute various clathrin-dependent phenotypes reported in this paper, thus excluding off-target effects (Figure S2).

In clathrin-KD cells, the internalization of EGFR, at low doses of ligand, was severely impaired, with a ~5- to 6-fold reduction in the internalization constant (K_e) (from 0.33 min^{-1} to 0.06 min^{-1}) (Figures 1A and 1B). At higher doses of ligand, however, the extent of the inhibition was significantly lower, around 2-fold, and the K_{eobs} decreased from 0.16 min^{-1} to 0.08 min^{-1} (Figures 1A and 1B). Thus, in keeping with our previous results (Sigismund et al., 2005), at low doses of EGF, EGFR was predominantly internalized by CME. However, at higher doses of ligand, NCE became substantial. From the kinetics shown in Figure 1A, we calculated that at high EGF ~60% and ~40% of the internalization events occurred through CME and NCE, respectively (see also Table 1, where a synopsis of all measurements performed in this study is reported). Importantly, the relative inefficiency of the clathrin KD to block internalization events under high EGF was not due to incomplete ablation, as shown by the fact that the internalization of another receptor, the Transferrin Receptor (TfR), whose internalization is completely clathrin-dependent, was almost completely abolished in the clathrin-KD cells (Figures 1A and 1B).

We next assessed the impact of clathrin KD on EGFR fate. We measured receptor degradation by following the disappearance of EGFR in intact cells exposed to EGF. To score the degradation of the receptor, in this kind of assay, we used high EGF. The rate of EGFR degradation was not markedly affected by functional ablation of clathrin (Figure 2A), although a small but reproducible increase in the half-life of EGFR, upon EGF stimulation, was observed (T/2 ~70 min and ~95 min in control and clathrin-KD cells, respectively, Figure 2A). Similarly, when we measured receptor downmodulation with ^{125}I-EGF, the kinetics of disappearance of EGFR from the cell surface were comparable in control and clathrin-KD cells (Figure 2B). Of note, the KD of clathrin did not have major effects on a panel of other endocytic proteins, arguing in favor of the specificity of the observed effects (Figure 2C).

A



B

	EGF LOW 1.5 ng/ml (Ke ± 95% CI)	EGF HIGH 20 ng/ml (Ke ± 95% CI)	EGF HIGH 100 ng/ml (Ke ± 95% CI)	EGFR (Rec/cell)	Tf (Ke)
Control	0.330 ± 0.011	0.163 ± 0.020	0.168 ± 0.023	4.4 x 10^5	0.63
Clathrin KD	0.062 ± 0.008	0.084 ± 0.010	0.075 ± 0.010	4.5 x 10^5	0.05
Filipin	0.315 ± 0.010	0.105 ± 0.011	0.106 ± 0.017	4.1 x 10^5	0.61
Clathrin KD + Filipin	0.058 ± 0.005	0.020 ± 0.003	0.021 ± 0.003	4.3 x 10^5	0.05
AP2 KD	0.170 ± 0.020	ND	0.134 ± 0.017	4.3 x 10^5	0.08
AP2 KD + Filipin	0.168 ± 0.015	ND	0.078 ± 0.015	4.2 x 10^5	0.08

Figure 1. EGFR Internalization in Various Conditions

(A) Kinetics of EGF internalization under the various indicated conditions. Results are the average of triplicate points (SEM < 10%) and representative of three independent experiments (see [B]).

(B) Internalization constants and receptor number/cell were measured under the indicated conditions. Note that at high EGF, the kinetic parameter does not measure the actual endocytic rate constant, hence the definition of observed K_e ($K_{e\text{-}obs}$, see also Wiley and Cunningham, 1982). Note also that, both at high and low EGF, 10%–15% of the internalization events were insensitive to the combined clathrin-KD and filipin treatment. This can be due to the existence of a pathway insensitive to these two conditions, or to an incomplete effect of the KD or of the drug treatment. We refer to this component operationally as "background internalization"; in all our calculations, this background was subtracted, in order to obtain parameters unequivocally ascribable to CME and NCE. Results are the average from three independent experiments ± 95% CI (confidence interval). ND, not done.

Table 1. EGFR Fate under Various Conditions

	Low EGF			High EGF		
	Total	CME (Filipin treated)	NCE (Clathrin KD)	Total	CME (Filipin treated)	NCE (Clathrin KD)
^{125}I-EGF internalization[a]	100%(85% CME+NCE)[d]	100%	0%	100%(90% CME+NCE)[d]	~60%	~40%
^{125}I-EGF degradation[b]	~30%	~30%	0%[e]	~55%	~30%	~80%–90%
^{125}I-EGF recycling[c]	ND			~40%	~60%–65%	~15%

Various parameters, concerning EGFR internalization and fate, are summarized.
[a] Data on ^{125}I-EGF internalization are extracted from the experiments presented in Figure 1.
[b] Data on ^{125}I-EGF degradation are extracted from the experiments shown in Figure 3A and 4C.
[c] Data on ^{125}I-EGF recycling are extracted from the experiments shown in Figure 5A. ND, not done; under low EGF the amount of internalized counts was too low and did not allow for a reproducible estimate of recycling rates.
[d] The indicated range of value refers to the subtraction of "background internalization" (see legend to Figure 1B); 100% means total internalization, 85% and 90% (in the case of low and high EGF, respectively) refer to the values unequivocally attributable to CME + NCE after subtraction of background internalization. For all other values reported, background internalization was subtracted before calculating percentages.
[e] The indicated value was not directly measured (due to the extremely low number of counts internalized under this condition), and it is inferred from the other calculations.

NCE Is Preferentially Coupled to EGFR Degradation

The above results indicate that CME does not play a major role in EGFR degradation and suggest that in order to be degraded the EGFR must be internalized through a clathrin-independent pathway. To gain more insights into this issue, we measured ligand degradation with ^{125}I-EGF.

As shown in Figure 3A, at low EGF (when CME is predominant), ~30% of the internalized ligand was degraded. At high EGF, ~55% of the ligand was degraded. Since under these conditions ~60% and ~40% EGF enters through CME and NCE, respectively, we calculated that ~90% of the EGF entering through NCE is destined to degradation (detailed calculations are in the legend to Figure 3A; see also Table 1).

To further strengthen our conclusions, we measured ligand degradation in clathrin-KD cells under high EGF. In this case, we detected increased ligand degradation with respect to control cells (Figure 3A). Under these conditions (in which CME is impaired), up to 80% of ^{125}I-EGF was degraded (Figure 3A), which is close to the 90% theoretically calculated above (see also Table 1). The degradation of NCE-internalized EGFR, under these conditions, was sensitive to the lysosomal inhibitor chloroquine (Figures 3B and 3C), demonstrating that NCE routes the receptor to lysosomal degradation. In addition, chloroquine exerted virtually indistinguishable effects on both control and clathrin-KD cells (Figures 3B and 3C), further confirming that efficient EGFR degradation requires receptor internalization through the NCE pathway.

Cholesterol-interfering drugs, such as filipin, can also be used to explore the NCE pathway. We find that filipin is preferable over other widely used drugs, such as β-methyl-cyclodextrin or nystatin, both for theoretical and experimental reasons (see Figure S3 for details). Filipin acts selectively on NCE-mediated internalization, as shown by its lack of effect on the internalization of EGFR under low EGF, or of the TfR (Figures 1A and 1B). However, at high EGF, filipin inhibited ~40% of all EGFR internalization events, in solid agreement with the amount of NCE-mediated internalization, measured in clathrin-KD cells (Figures 1A and 1B, Table 1). Indeed, under conditions of high EGF, near-complete inhibition of internalization could be achieved only by combining clathrin-KD and filipin treatment (Figures 1A and 1B). We further note that the existence of two routes of internalization of the EGFR, and their selective sensitivity to clathrin-KD

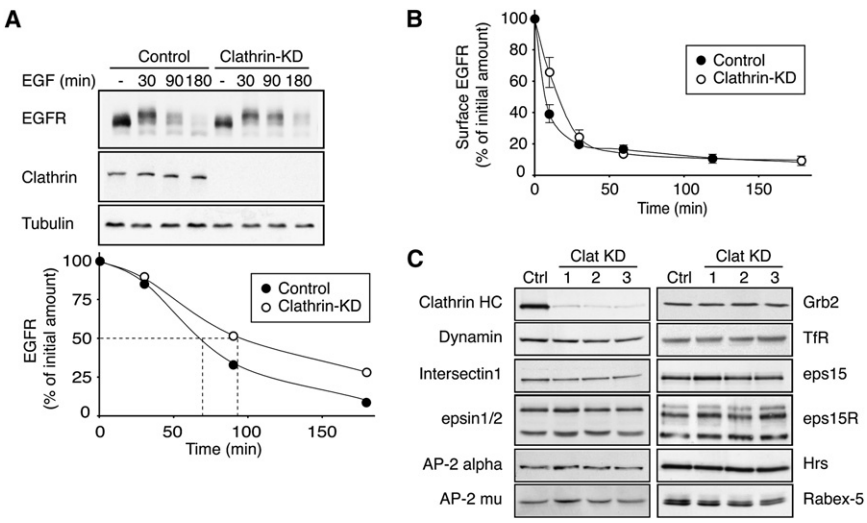


Figure 2. EGFR Degradation and Downmodulation in Clathrin-KD Cells

HeLa cells were subjected to clathrin KD, as indicated, or to transfection with control oligo (mismatch of oligo 1, Ctrl).

(A) Receptor degradation. IB was as shown. (Bottom panel) Densitometry of EGFR blot. Results are typical and representative of at least three experiments.

(B) Receptor downmodulation. Results are the average from three independent experiments ± 95% CI.

(C) HeLa cells were subjected to clathrin KD, with three different silencing oligos, or to transfection with control oligo (mismatch of oligo 1, Ctrl). The expression of various endocytic proteins was tested as shown.

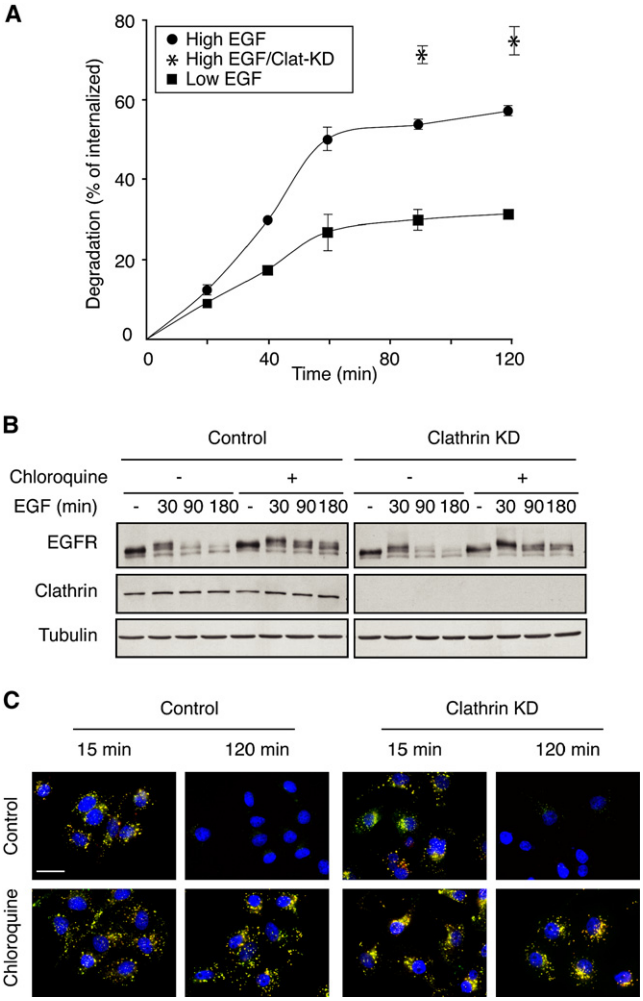


Figure 3. NCE-Mediated Degradation of EGFR

(A) ^{125}I-EGF degradation. High EGF, 20 ng/ml; low EGF, 1.5 ng/ml. Results are the average from three independent experiments ± 95% CI. Ligand degradation in clathrin-KD cells was measured at 90 and 120 min, since the overall reduced internalization, under this condition, did not yield sufficient counts for reliable measurements at shorter times. Data were obtained after subtraction of values obtained (in parallel experiments) under conditions of clathrin-KD/filipin treatment to remove "background internalization" (see Figure 1). These values never exceeded 10%–20% of the total counts. Calculation of the fraction of NCE-internalized EGFR undergoing degradation: under high EGF ~55% EGF is degraded, and ~60% and ~40% EGF enters through CME and NCE, respectively. Since ~30% EGF entering through CME is degraded, the CME pathway contributes ~18% (30% of 60%) to degradation of EGF, under high EGF. The remaining 37% degraded EGF (55%–18%) is attributable to NCE. Since 40% EGF enters, under high EGF, through this route, ~90% (37/40 = 0.925) of the EGF entering through NCE is destined to degradation.

(B) Cells were treated with chloroquine (100 μM for 3 hr at 37°C) and EGF (100 ng/ml) as indicated. IB was as shown.

(C) Cells were treated with chloroquine as shown and incubated with anti-EGFR (green, 13A9) and rhodamine-EGF (red) for the indicated times. Note that after 15 min of EGF treatment, ligand and receptor signals colocalized in endosomes, in both control and clathrin-KD cells. At 120 min, nearly all signals disappeared in cells not treated with chloroquine, suggesting degradation. Conversely, in chloroquine-treated cells, signals persisted in vesicular compartments and no significant differences were detected between control and clathrin-KD cells. Blue, DAPI. Bar, 18 μm.

or to filipin treatment, could be confirmed in three independent populations of HeLa cells (data not shown, see the Experimental Procedures) and also in the fibroblastic cell line NR6 (see below).

When we measured EGF degradation in the presence of filipin under high EGF, a condition under which NCE is inhibited, we detected a conspicuous inhibition of EGFR degradation (Figure 4A), which correlated well with the kinetics of disappearance of EGFR from the surface under the same conditions (Figure 4B). In this latter assay, in filipin-treated cells after an initial disappearance, the receptor reappeared on the surface, suggesting increased recycling (Figure 4B).

We then measured ligand degradation with ^{125}I-EGF. Under conditions of high ligand, the degradation of EGF was dramatically reduced in the presence of filipin (Figure 4C). Actually, the kinetics of degradation of high EGF in filipin-treated cells were virtually identical to those of low EGF in control cells, further reinforcing the notion that NCE is required for efficient EGFR degradation (see also Table 1). Of note, filipin treatment did not have any measurable effect on the degradation of EGF at low doses of ligand (Figure 4C). This result demonstrates that filipin does not interfere with the degradation of EGFR when the receptor is internalized through CME. It should be further added that filipin did not interfere with the degradation of low density lipoprotein (LDL) or of bovine serum albumin (BSA), which represent prototypes of molecules internalized through CME or fluid phase endocytosis, respectively, and which are normally destined to lysosomal degradation (Figure S4). Thus, filipin does not interfere with steps of endocytosis that are distal to internalization.

In summary, through two different approaches, based on molecular genetics (clathrin KD) and pharmacological interference (filipin treatment), we demonstrated that NCE is the preferential mode of internalization that destines the EGFR to degradation.

CME Sustains EGFR Recycling to the Cell Surface

Since we established that EGFRs internalized through CME are committed to degradation in only 30% of the cases (Table 1), we questioned what the fate of the remaining 70% EGFRs might be. One possibility is that the EGFR internalized through this route is recycled to the plasma membrane. To test this hypothesis, we measured recycling in the presence of high EGF with two different methods.

First, we followed ligand recycling using ^{125}I-EGF. In control HeLa cells, ~40% of EGFR was recycled (Figure 5A, Table 1). This result is congruent with the 55% degraded EGF that we observed under the same conditions (Figure 3A, Table 1). The impairment of CME, through clathrin KD, caused a significant decrease in the recycling kinetics (corresponding to an ~2-fold reduction in the recycling rate; data not shown). In clathrin-KD cells only ~15% of the EGFR was recycled to the plasma membrane (Figure 5A, Table 1), in excellent concordance with the estimated 80%–90% EGFR degraded under the same conditions (Figure 3A, Table 1). By applying the same logic used in Figure 3A to calculate the extent of receptor degradation, we further calculated that ~60% of the receptor internalized through CME is recycled, a value in good agreement with the 30% degradation associated with this route (Figure 3A). Treatment of HeLa cells with filipin further confirmed these quantitative assessments, as ~65% of the receptors internalized under these conditions were recycled to the surface (Figure 5A, Table 1).

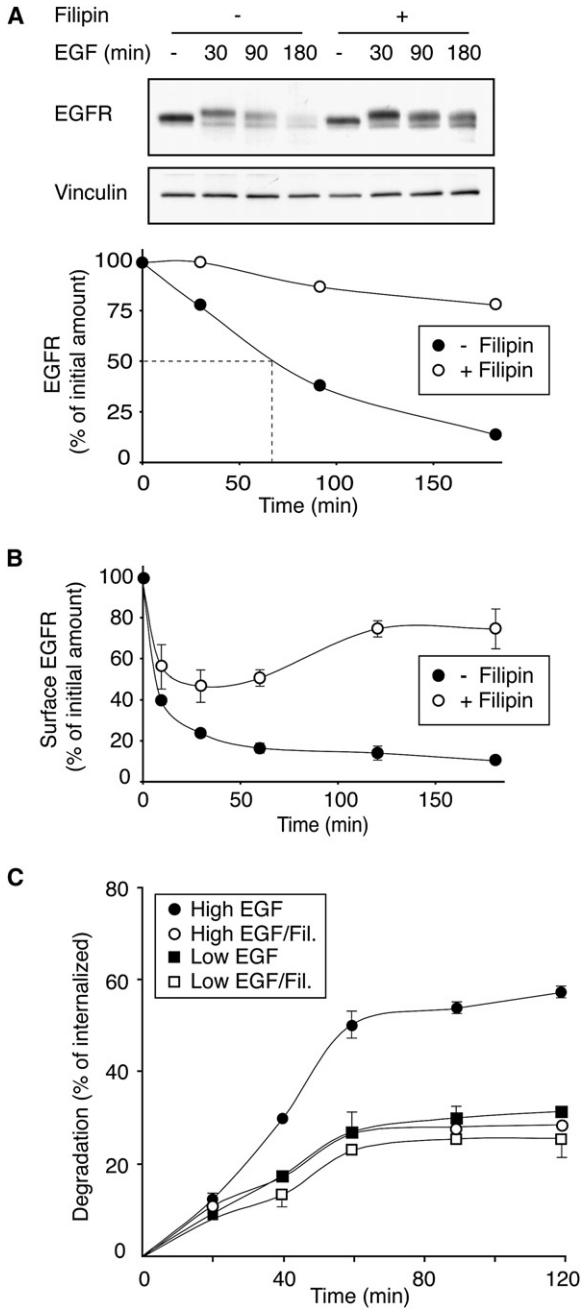


Figure 4. EGFR Degradation and Downmodulation in Filipin-Treated Cells

(A) Receptor degradation. HeLa cells were treated with filipin or mock-treated. IB was as shown. Bottom panel, densitometry of EGFR blot.

(B) Receptor downmodulation. Results are the average from three independent experiments ± 95% CI.

(C) ^{125}I-EGF degradation. The experiment was performed as in Figure 3A, including subtraction of "background internalization." Results are the average from three independent experiments ± 95% CI. The "– filipin" curves are the same as in Figure 3A, since all measurements come from the same experiments.

Finally, independent measurements by a FACS-based assay also showed a sizable decrease in EGFR recycling in clathrin-KD cells compared to control cells, which was mirrored by an equally evident increase in filipin-treated cells (Figure 5B, see also Figure S5A for additional experiments). Of note, filipin did not affect the recycling rate of the TfR, which is internalized exclusively through CME, thus excluding nonspecific effects of the drug on recycling (Figure S5B).

As an additional tool, we employed a mutant EGFR, Y1045F, in which mutagenesis of a single Tyr dramatically reduces receptor ubiquitination (Figure S6A; Levkowitz et al., 1999). Since we previously showed that EGFR ubiquitination is indispensable for NCE, but dispensable for CME (Sigismund et al., 2005), we predicted that EGFR-Y1045F would be internalized almost exclusively through CME. In addition, since this mutant cannot be ubiquitinated, it should not be routed to late endosomal stations and might be recycled back to the cell surface with high efficiency. We used NR6 fibroblasts, devoid of EGFR (Pruss and Herschman, 1977), to perform experiments with the Y1045F mutant. We expressed either EGFR wt or the Y1045F mutant at comparable levels in these cells (Figure S6B). Although the kinetics of internalization of the EGFR displayed some difference with respect to HeLa cells (see Figures S6B and S6C for details), as expected the Y1045F mutant was internalized almost exclusively through CME, both at low and high EGF (Figures S6B and S6C). In addition, EGFR-Y1045F displayed reduced degradation and increased recycling when compared to the wt receptor (Figures S6D and S6E). Of note, ~70% of the Y1045F mutant was recycled to the cell surface. Since these latter results were obtained in the absence of functional ablation of components of the clathrin pathway, or of pharmacological interference, they show that the CME pathway possesses the intrinsic ability to sustain high rates of recycling of the EGFR.

Intracellular Trafficking of NCE-Internalized EGFRs

On the basis of the above experiments, we concluded that the EGFR undergoes profoundly different fates, depending on the engaged pathway of internalization (CME versus NCE). CME-internalized receptors are predominantly recycled to the cell surface and only a fraction of these receptors is committed to degradation. Conversely, NCE-internalized receptors are predominantly destined for degradation and they show little, if any, recycling to the plasma membrane. Current knowledge holds that internalized EGFR is routed to early endosomes, where its fate (recycling or degradation) is decided. An obvious question, therefore, is whether NCE-internalized receptors are trafficked through the canonical endosomal pathway or through a different route. To gain insights into this issue, we investigated the colocalization of rhodamine-EGF with endosomal (early, late, recycling) and lysosomal markers. We performed these experiments in clathrin-KD cells under high EGF, a condition that allows for the quasi-selective exploration of NCE. We found that EGF colocalized with EEA-1 (early endosomes) comparably in clathrin-KD and control cells (Figure 5C). As expected, based on all previous evidence, EGF showed increased colocalization with late endosomes (Rab7) and lysosomes (Lamp2), and decreased colocalization with a marker of recycling endosomes (Rab11) in clathrin-KD cells. These results, coupled with the previous demonstration that NCE-internalized EGFRs are degraded in the lysosome (Figures 3B and 3C), indicate NCE-internalized receptors are trafficked through a canonical endosomal route. Finally, as expected from the results shown so far, EGF

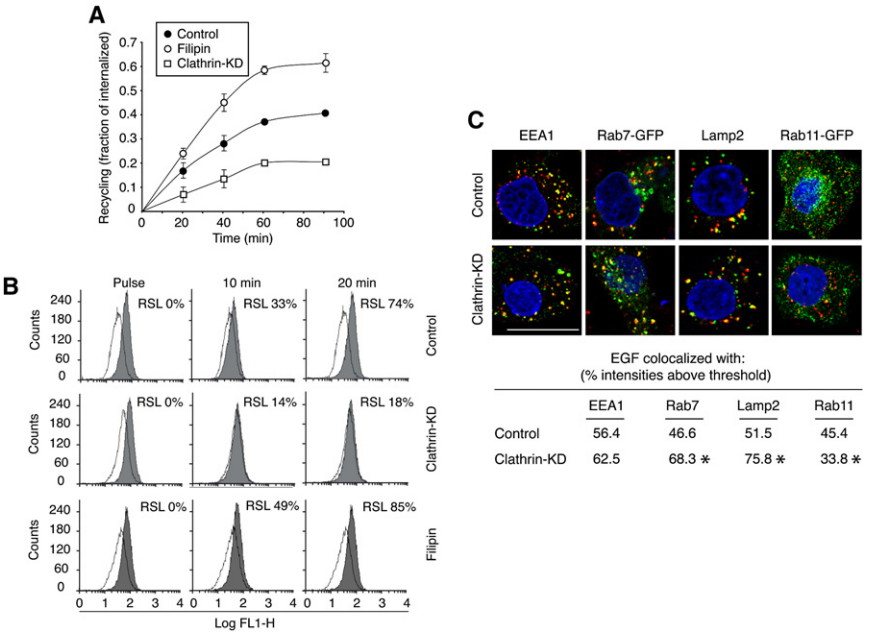


Figure 5. EGF and EGFR Recycling and Degradation

(A) ^{125}I-EGF recycling. Results are the average from three independent experiments ± 95% CI.

(B) EGFR recycling measured by FACS. "Pulse," surface EGFR was measured in starved cells, before (gray area) or after a 15 min pulse of EGF (100 ng/ml, white area). Panels "10 min" and "20 min," surface EGFR was measured (gray areas) 10 and 20 min after the pulse (white areas, surface EGFR at pulse time). RSL, relative surface level (see the Supplemental Experimental Procedures).

(C) Colocalization of EGF and various endocytic markers. HeLa cells (transfected where indicated with Rab7-GFP or Rab11-GFP), were subjected to control siRNA (control) or clathrin KD. Cells were then incubated with rhodamine-EGF (0.5 μg/ml, red) for 10 min (for the detection of colocalization with EEA1) or 30 min (for the detection of colocalization with Rab7, Rab11, Lamp-2), and stained (green) for EEA-1 or Lamp-2; Rab7 and Rab11 were detected by epifluorescence. Merged channels are shown. Bar, 18 μm. Results are typical and representative of three experiments. The percent of colocalization of rhodaminated-EGF with the various markers (% intensities above threshold) is also shown; *, $p < 0.05$ with respect to "control."

internalized through CME (filipin treatment) showed reduced colocalization with markers of late endosomes and lysosome, and increased colocalization with Rab11 (Figure S5C).

CME Sustains Prolonged EGFR Signaling

We have demonstrated that CME does not represent a major mechanism of EGFR attenuation, but its physiological role remains undefined. Endocytosis has been linked to both signaling attenuation and to signaling enforcement; thus, we investigated the impact of CME (and of NCE) on EGFR receptor signaling. As before, our experimental strategy relied on the fact that functions connected with CME can be explored in HeLa cells stimulated with low EGF, whereas NCE-associated phenotypes can be investigated in clathrin-KD cells stimulated with high EGF. As shown in Figure 6, both under low or high EGF, the kinetics of phosphorylation of different substrates (AKT, ERKs, SHC) in control HeLa cells were biphasic, with a rapid peak (5 min) followed by a slow decay.

In the case of P-AKT (and to a lesser extent also in the case of P-ERKs), clathrin KD did not have much effect on the peak phase (some reduction was observed at low EGF), but severely dampened the decay phase (Figures 6A and 6B). Thus, prolonged signaling requires CME for certain pathways. A straightforward interpretation of these results is that the peak phase of signaling is endocytosis-independent (probably exclusively originated at the plasma membrane), while a sustained decay phase requires CME. In the case of P-SHC, conversely, the kinetics of phosphorylation were not disturbed by clathrin KD (Figures 6A and 6B), arguing for their CME-independence.

A corollary of the above findings is that under conditions in which NCE is inhibited, the kinetics of phosphorylation of substrates such as AKT or ERKs should be altered and should display a sustained decay phase. Indeed, when HeLa cells were treated with filipin and high EGF, there was increased phosphorylation of AKT and ERKs, during the decay phase, with no effect on the peak phase (Figures 6C and 6D). Importantly, under low EGF (a condition in which CME is preponderant), filipin did not alter the kinetics of phosphorylation of AKT and ERKs, thus excluding nonspecific effects of the drug. As expected, based on the previously described results, the kinetics of P-SHC were not altered by filipin treatment (Figures 6C and 6D).

To further corroborate these conclusions, we used the Y1045F mutant, which by being continuously recycled to the cell surface, and being subjected to very limited degradation, should phenocopy the effects of filipin on EGF-activated signaling. Indeed, as shown in Figures 6E and 6F, EGF stimulation of Y1045F resulted in an enhancement of the decay phase of phosphorylation of AKT (and to a lesser extent of ERKs), in the absence of noticeable alterations of the peak phase. Importantly, filipin treatment did not further enhance signaling by the Y1045F mutant (Figure 6E), thus arguing that, in accordance with the initial hypothesis, the Y1045F mutant phenocopies the signaling effects of filipin.

CME-Mediated Signaling Is Necessary for the Biological Functions of EGFR

One important caveat to address, in the interpretation of the above results, concerns a possible signaling role of clathrin. Emerging evidence, indeed, points to a function of clathrin in the assembly of signaling complexes at the endosome, as well as to a direct role in transcription (reviewed in Mills, 2007). Thus, it is important to assess whether the signaling effects of clathrin KD are due to the impairment of CME or to the perturbation of other "signaling" functions of clathrin.

To gain insights into this issue, we interfered with CME by an alternative approach, i.e., through the ablation of AP-2. We

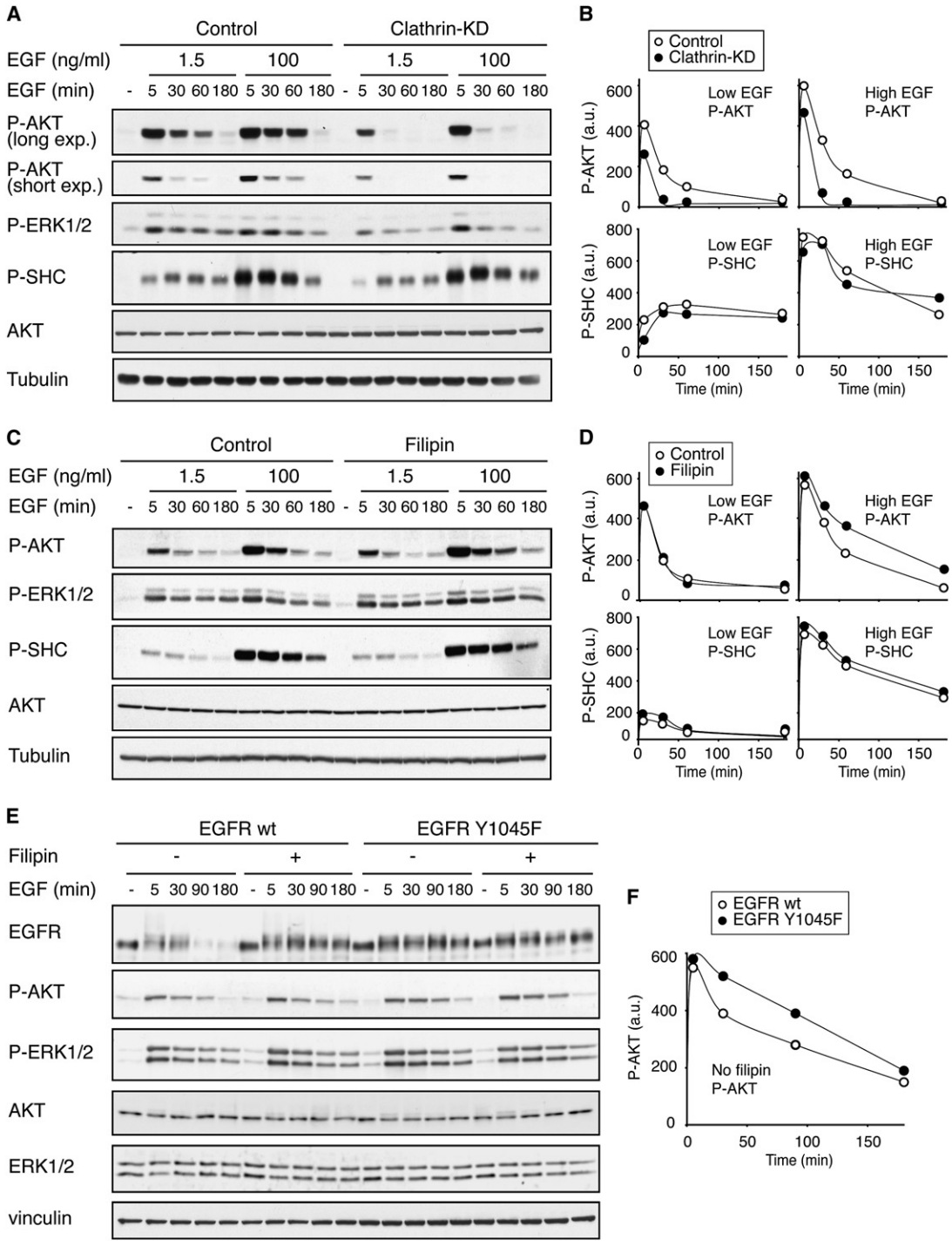


Figure 6. CME- and NCE-Dependent EGFR Signaling

(A–D) HeLa cells were subjected to clathrin KD (A) or treated with filipin (C), and treated with EGF as indicated. IB was as shown. In (B) and (D), a semiquantitative evaluation of (A) and (C), respectively, is reported. Densitometry was performed on different exposures of the blots and average results are reported, as arbitrary unit (a.u.) for P-AKT and P-SHC. The half-time of the decay of the P-AKT signals was also estimated in control cells, under low or high EGF, in four independent experiments and yielded the following results: low EGF, 25–30 min, high EGF 35–40 min.

(E and F) NR6 cells, transfected with wt EGFR or the Y1045F mutant, were treated with EGF (100 ng/ml) as indicated. IB was as shown (E). Results are typical and representative of at least three experiments. A densitometric analysis (performed as in [B] and [D]) is reported in (F).

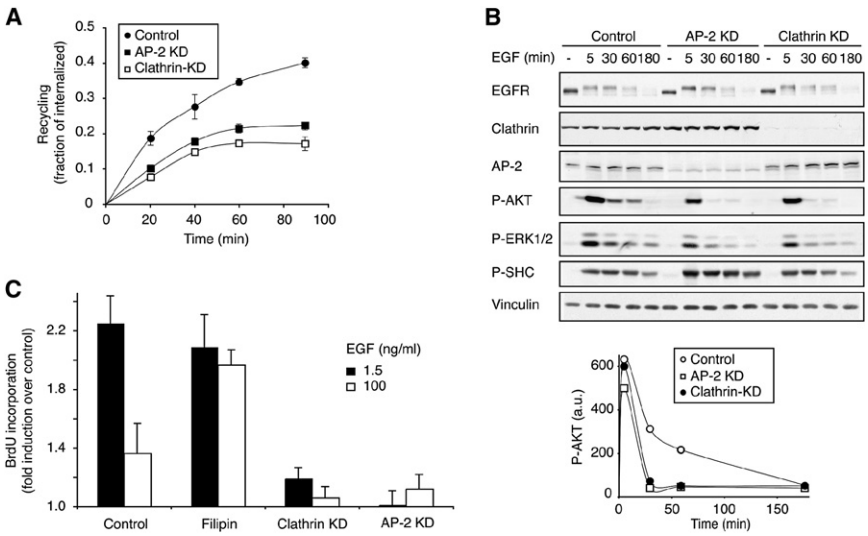


Figure 7. The KD of AP-2 Phenocopies the Clathrin KD

HeLa cells were or subjected to AP-2- (α subunit) or clathrin KD.

(A) ^{125}I-EGF recycling. Results are the average from three independent experiments ± 95% CI.

(B) KD cells, treated as shown, were IB with the indicated Abs. Results in all panels are typical and representative of at least three experiments. A densitometric analysis of P-AKT signals is reported in the lower panel.

(C) DNA synthesis was monitored by BrdU incorporation. Results are expressed as fold induction, normalized to incorporation in serum-starved cells not treated with EGF (assumed as 1). Results are the average from three independent experiments ± 95% CI.

performed KD of the α subunit (Table 1, Figure 7) or of the μ subunit (data not shown) with comparable results. Of note, no direct "signaling" functions have been described for AP-2. The KD of AP-2 reduced the CME component of EGF internalization, while leaving the NCE component unaltered (Table 1). As previously reported (Huang et al., 2004), the effects on EGF internalization of the AP-2 KD were of a lesser magnitude with respect to the clathrin KD. This was not due to inefficient ablation (see effects on Tf internalization, Table 1), and probably depends on the existence of alternative adaptors. However, the AP-2 KD reduced EGF recycling as efficiently as the clathrin KD (Figure 7A) and did not significantly affect EGFR degradation (Figure 7B, panel EGFR). Importantly, when signaling was analyzed, we detected in AP-2-KD cells the same alterations in the kinetics of P-AKT (and of P-ERK1/2) as in clathrin-KD cells, while P-SHC kinetics were unaffected (Figure 7B). Thus, two independent ways of affecting CME (clathrin KD or AP-2 KD) have the same effects on signaling.

The availability of AP-2-KD cells (in addition to clathrin-KD cells) also allowed us to explore the impact of CME on biological endpoints of EGF stimulation. We monitored EGF-induced DNA synthesis. Both the KDs of AP-2 and clathrin substantially reduced this biological endpoint, when compared to control cells, both at high and low EGF doses (Figure 7C). Thus, CME is required not only to sustain short-term EGFR signaling (Figure 6), but also for its long-term biological effects.

It is well known that EGF-stimulated DNA synthesis and mitogenic response exhibit a bell-shaped dose-response curve, in which low concentrations of EGF have stronger effects than higher concentrations (see for instance Di Fiore et al., 1987). This is probably due to the fact that high doses of EGF induce acute downmodulation of the receptor. We reasoned that, under conditions of filipin treatment in which only CME is active, this effect should be reverted. As shown in Figure 7C, in control HeLa cells, low EGF was ~3-fold more efficient at inducing DNA synthesis than high EGF. However, in the presence of filipin treatment, low and high concentrations of EGF were equally potent. These results support the idea that CME is required for sustained signaling and also provide a mechanistic framework for the different biological outcomes observed when challenging cells with different concentrations of EGF under physiological conditions.

DISCUSSION

Integration of Different Endocytic Routes in EGFR Signaling and Attenuation

Our results highlight an unexpected picture of how endocytosis regulates EGFR signaling and attenuation. At low EGF, when CME is predominant, a large fraction of the receptor (roughly two thirds) is recycled to the cell surface and only one third of EGFR is destined to degradation. This, in turn, allows for prolonged CME-dependent signaling. At high EGF, while CME continues to be operational, NCE kicks in and destines a sizeable part of the receptor to degradation, thereby protecting the cell from overstimulation. Interestingly, this dual mechanism seems to be well designed to cope with the vast variety of physiological EGF concentrations, which range from fractions to hundreds of nanograms per ml, in bodily fluids (reviewed in Sigismund et al., 2005).

While somehow surprising, it is important to note that our results do not contradict any extant body of knowledge. Under our conditions of analysis, the EGFR is internalized with K_e similar to those reported in the literature (see for instance Huang et al., 2004), and it is "canonically" routed to early endosomes. Then it is either recycled to the cell surface (with kinetics comparable to previously reported ones; see for instance Kornilova et al., 1996) or destined to lysosomes for degradation (with half-life comparable to the previously reported one; see for instance Stoscheck and Carpenter, 1984). However, we provide a new outlook on how these events are achieved through the combined (and by-and-large opposing) action of two different internalization mechanisms. We performed a thorough quantitative analysis of EGFR internalization, degradation, recycling, and signaling, under conditions in which we selectively allowed only one of the two pathways (either CME or NCE) to be preferentially operational in any given experiment. When added up, the numeric values obtained under these selective conditions

(see Table 1) matched those obtained in control cells remarkably well, at various doses of EGF, demonstrating significant internal consistency. Thus, through the dissection of the relative contribution of CME and NCE, we identified specific functions executed by the two pathways, which were previously attributed globally to the clathrin-mediated pathway alone.

CME and Signaling

Our results indicate that the major function of EGFR-CME is to sustain signaling. This seems to be a global property of the pathway, rather than the result of putative signaling properties of some of its components, since comparable results were obtained using two different molecular tools, the KD of clathrin and that of AP-2. Our detailed kinetic analysis revealed that some signaling pathways (AKT and ERK) require intact CME, while others (SHC) do not. In addition, the execution of complex biological functions, such as EGFR-activated DNA synthesis, depends on functional CME. The sum of our results is consistent with the interpretation that the initial phase of signaling (the peak phase) is endocytosis-independent, probably occurring at the plasma membrane, while later events (the decay phase) require CME. In turn, this is perfectly compatible with the fact that CME sustains recycling, a function that is expected to have a delayed impact on phosphorylation kinetics.

An important role of internalization in signaling is well documented (von Zastrow and Sorkin, 2007). This has been largely attributed to the ability of endosomes (the so-called ''signaling endosomes'') to serve as an intracellular platform, conferring signal specificity and diversity, possibly by extending and segregating the repertoire of regulatory molecules and/or effectors (see for instance Mor and Philips, 2006). Our results point to an additional mechanism, through which CME contributes to signaling, i.e., recycling. Recycling can impact on signaling in two ways. Continuous recycling of receptors to the plasma membrane would allow for multiple cycles of signaling from both the plasma membrane and the endosome, preserving the receptor from degradation: a mechanism that could be particularly advantageous under conditions of limited ligand availability. Recycling might also permit the relocalization of the receptor (or of associated signaling machinery) to regions of the plasma membrane where spatially restricted signaling is required for events such as actin dynamics and cell motility. Indeed there is growing evidence that endocytosis is required to ensure the spatial resolution of stimuli originating from motogenic receptors, and to orchestrate localized actin dynamics, polarized protrusive activity, and directed cell motility (Emery and Knoblich, 2006; Jekely et al., 2005; Marco et al., 2007; Wang et al., 2006).

Sorting of the EGFR toward Degradation or Recycling

While the majority of CME events lead to recycling, a fraction of them (~30%) appear to commit the EGFR to degradation. One might ask, therefore, whether this is stochastic or determined mechanistically, for instance through molecular differences between the coated pits that internalize EGFR destined to recycling or to degradation. The proposition is not implausible, since recent results suggest the existence of cargo-specific CME routes [see for instance (Puthenveedu and von Zastrow, 2006; Sorkin, 2004; Tosoni et al., 2005)]. The question is whether an individual cargo species, such as EGFR, can be internalized through molecularly different (at least in part) coated pits, and if this has functional consequences. We note that functional ablation of AP-2, the major clathrin adaptor, only abrogates ~50% of CME-dependent EGFR internalization (as measured at low EGF). This is in line with previous results (Huang et al., 2004) and with the existence of alternative adaptors. However, the perturbations induced by the AP-2 KD on recycling and signaling were quantitatively very comparable to those induced by the clathrin KD, suggesting that the fraction of CME that is AP-2-dependent is the one associated with recycling and recycling-dependent signaling. In turn, this allows speculation that EGFRs internalized through coated pits formed by adaptors other than AP-2 might be those subjected to CME-dependent degradation (Lakadamyali et al., 2006). The issue of whether different EGFR-internalizing pits are coupled with different functional outcomes appears, therefore, worthy of future investigations.

A related issue concerns the trafficking pattern of the EGFR, internalized through CME or NCE. We were able to ascertain that EGFRs internalized through either pathway were routed to early endosomes. It remains to be established how they are differentially sorted to recycling (in the case of CME) or to degradation (in the case of NCE). One obvious possibility stems from evidence that clathrin might be required for endosomal sorting and recycling (Newmyer and Schmid, 2001; van Dam and Stoorvogel, 2002), a function that might be related to the demonstrated assembly of endosomal coats containing clathrin and Hrs (Raiborg et al., 2002). Thus, sorting of EGFR at the endosome might also be partially clathrin-dependent, and sensitive to clathrin KD. However, the KD of AP-2 phenocopied the KD of clathrin, as far as sorting to a recycling fate was concerned. Of note, there is no evidence that AP-2 is involved in endosomal sorting. Thus, alternative possibilities should also be considered. One such possibility is that molecular determinants carried by CME- or NCE-originated vesicles affect sorting in endosomes. Clearly, more work will be needed to distinguish among these and other hypotheses.

The Nature of the NCE Pathway

Our results establish that the major function of NCE is to commit the EGFR to degradation. This was established through two approaches: by analyzing a series of parameters under conditions in which NCE was preferentially active (in clathrin-KD cells) or by inhibition of NCE with filipin. The two approaches yielded quantitatively complementary results, thus providing internal validation. In addition, the extent of EGFR degradation scored in the presence of filipin and under high EGF was almost identical to that detected in control cells treated with low EGF (in the absence of the drug). Therefore, two distinct ways to explore selectively the CME pathway yielded the same results.

Much remains to be understood. Our results, while shedding light on the role of NCE in EGFR physiology, do not address its molecular mechanisms. We would like to expressly state that nothing in our data should be taken to indicate support for a role of caveolae in EGFR-NCE. We have shown that under high EGF there is increased localization of the EGFR to caveolae (Sigismund et al., 2005). This, however, does not imply that NCE occurs through them. Indeed, we show here that NCE of EGFR is caveolin-1-independent (see Figure S7, where literature in disagreement with our findings [Kazazic et al., 2006] is also

discussed). In addition, our data do not resolve whether EGFR-NCE is a single pathway or a combination of pathways. Regardless, we note that even if multiple pathways should contribute to NCE, their nature seems to be cumulatively degradative.

Our results clearly establish that, in the case of the EGFR, the recycling/signaling and the degradative fates are preferentially associated with different endocytic routes. This raises one final question: are these generalized properties of CME and NCE? We note that in the case of the TGF-β receptor a situation similar to that described here has been reported (Di Guglielmo et al., 2003). However, until a clear molecular picture of NCE is acquired, caution is appropriate and conclusions should remain restricted to the model under experimental scrutiny.

EXPERIMENTAL PROCEDURES

Chemicals, Antibodies, and Cells

A detailed list of all chemicals and antibodies used is in the Supplemental Experimental Procedures. HeLa or NR6 cells were transfected with FuGENE 6 (Roche) or OligofectAMINE (Invitrogen). For filipin treatment, cells were serum-starved for 4 hr and then preincubated for 1 hr with 0.5–1 μg/ml of filipin (SIGMA), followed by the indicated experimental procedures. In each experiment, internalization of ^{125}I-Tf was measured (data not shown), to exclude nonspecific effects. The experiments reported in this study were performed on an in-house population of HeLa cells. However, results on internalization and receptor degradation were confirmed on two additional populations of HeLa cells, one purchased from ATCC and one corresponding to the isolate HeLa "Kyoto" (Neumann et al., 2006).

Biochemical Assays

Lysis and immunoblotting (IB) were as described (Penengo et al., 2006). Quantification of blots was performed with ImageJ.

EGFR total surface levels were measured by saturation binding assays with iodinated ligands as described (Tosoni et al., 2005).

Internalization of ^{125}I-EGF and ^{125}I-Tf, and recycling and degradation of ^{125}I-EGF, were performed as described (Haglund et al., 2003; Kornilova et al., 1996; Sorkin et al., 1991a, 1991b; Tosoni et al., 2005). Additional details are in the Supplemental Experimental Procedures.

For the downmodulation assay, cells were serum-starved for 4 hr and then incubated with EGF (100 ng/ml) at 37°C for the indicated time points. Cells were then subjected at 4°C to "mild acid/salt treatment" (0.2 M Na Acetate buffer [pH 4.5], 0.5 M NaCl) to remove bound EGF, followed by measurement of surface EGFR by saturation binding with ^{125}I-EGF.

Functional Ablation of Clathrin or AP-2

Silencing of clathrin heavy chain or of AP-2 in HeLa cells was by transient transfection of siRNA oligos (from Dharmacon or Invitrogen) as described (Motley et al., 2003). Three different RNAi oligos (sequences are in the Supplemental Experimental Procedures) were used to target the clathrin heavy chain. For each oligo, a mismatched control was designed by introducing at least four mutations in the sequence (see the Supplemental Experimental Procedures). In each assay, at least two (of three) targeting oligos were used with comparable results.

Methodologies for reconstitution of clathrin in KD cells and for the silencing of clathrin in NR6 cells are in the Supplemental Experimental Procedures.

Two different RNAi oligos were used to target AP-2 (from Dharmacon, sequences are in the Supplemental Experimental Procedures). In each assay, the two oligos yielded comparable results.

Colocalization Studies

Internalization assays of fluorochrome-conjugated ligands and immunofluorescence analysis were performed as described (Haglund et al., 2003; Tosoni et al., 2005). The ImageJ software (W. Rasband, National Institutes of Health, Bethesda, MD, USA) was used for quantitative analysis on an average of 6–8 cells per condition. Background intensity was initially subtracted by placing "Regions of Interest" (ROI) over areas devoid of specific signal. The colocalization analysis plug-in (T. Collins, WCIF, Toronto Western Research Institute, Toronto, Canada) provided the reported percentage of colocalized intensity for each channel. Colocalizing intensity pixels were calculated above a specific intensity threshold (subsequently normalized to total channel intensity) giving the reported % intensities colocalized above threshold.

BrdU Incorporation Studies

HeLa cells (50% confluency) were serum starved for 24 hr and pretreated with filipin (0.5 μg/ml for 30 min at 37°C) or mock treated, followed by incubation with EGF for 8 hr at 37°C, in the presence or absence of filipin. BrdU (33.3 μM) was then added for 30 min. Detection was with an anti-BrdU antibody (Becton Dickinson). Additional details are in the Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and seven figures and are available online at http://www.developmentalcell.com/cgi/content/full/15/2/209/DC1/.

ACKNOWLEDGMENTS

We thank Stephen J. Royle, Sylvie Urbè, Marino Zerial, and GENENTECH for reagents; and Pietro De Camilli, Alexander Sorkin, and Pascale R. Romano for critically reading the manuscript. This work was supported by grants from AIRC (Italian Association for Cancer Research) to S.P. and P.P.D.F., The European Community (VI Framework) to P.P.D.F.; AICR (Association for International Cancer Research) to S.P.; and the Monzino and Ferrari Foundation to P.P.D.F.

Received: April 12, 2008
Revised: June 11, 2008
Accepted: June 20, 2008
Published: August 11, 2008

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